*i*Journals: International Journal of Software & Hardware Research in Engineering (IJSHRE)
ISSN-2347-4890
Volume 10 Issue 3 March 2022

Inhibitory Activity of Citrullus Colocynthis Extract on Native Leukemic Stem Cells

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ABSTRACT

The failure of classical anti-leukemic drugs for treatment leukemia led to relapse. The majority of leukemic patients achieve complete remission after intensive chemotherapy, the majority relapse after 5 years and die of disease. Relapse still occurs in 80% of patients.

Objective: The present study aimed to evaluate the cytotoxic activity of ethanolic extract from *Citrullus colocynthis* "L" fruit pulp against LSCs aspirated from native bone-marrow relative to classical anti-cancer drug vencrstine in a short term culture.

Methodology: *Citrullous colocynthis* fruit pulp extract of 10 mg/ml in ethanol 80% subjected to culture cells of LSCs density with 1 x 10^6 cells/ml. The major compounds of fruit pulp extract *C. colocynthis* were screened by standard method of analysis. At source of LSCs, freshly aspirated native bone marrow samples were collected from three newly diagnosed

Two of the patients had acute myeloid leukemia (AML) and the third one had, acute lymphoid leukemia (ALL). Density-gradient method was used for separation the blast cells. Isolated of bone marrow material were treated with fruit pulp extract of *C. colocynthis*. Vencrstine was used as positive control, whereas negative control remained cell culture without treatment. Cell viabilities tests were performed according to protocol of flowcytonctric method using 7-aminoactinomycin D. (7.A.A. D) to label dead cells.

Results: Different among treatment and control samples were found to be highly significant P<0.001. Fruit pulp extract gave 13.5%, 21.3% and 29.5% cell death for sample 1,2 and 3 respectively, whereas vencrstive gave mortality rates of 2.3%, 6%% and 6% for sample 1, 2 and 3 respectively, when control negative scored 2.2%, 5.8% and 5.8% cell death for sample 1, 2 and sample 3 respectively.

Conclusion: Our findings suggest that fruit pulp extract from *C. colocynthis* induced apoptosis against LSCs on all types and subtypes of leukemia, and dose not harm normal stem cells and it can be one of selective target for LSCs.

Keyword :*Citrullus colocynthis* ,leukemic stem cells ,bone marrow , vencrstine ,flowcytometry , lymphoid leukemia, myeloid leukemia high bone marrow blast cells.

INTRODUCTION

The American Cancer Society, estimates for AML in the United States for the year 2020 are about 19, 940 new cases and 11, 180 death.

The standard classical anti-leukemic treatment for decades ago has not changed and it consist of intensive combined therapy with cytarabine plus anthracycline (Dohner *et al.*, 2010).Data and evidence accumulated that cancer cells where driven by subpopulation of stem cells and gives rise to cancer population.

Approximately up to 70% adults patients and 30% of children will not survive more than 5 years after initial treatment due to relapsing disease (De Rooij *et al.*, 2015).

(B.Bose *et al.,* 2017) reported that the majority of leukemic patients achieve complete remission after treatment. However subsequently relapse occurs after 5 years in 80% of patients die of disease .

Classical treatment of cancer have shown limited effect on advantage stages of disease because the treatment are especially target tumor bulk cells rather than target stem cancer cells (Reya *et al.,* 2001).

The Failure of classical anti-cancer drugs due to quiescent state of stem cells, and the cytotoxic drugs acting only on replicating cells (Ishikawa *et al.,* 2007) (Costello *et al.,* 2000).

First described stem cells in leukemia (Bonnet and Dick, 1997).The optimal treatment is depending on eradicate LSCs revealed that (Dick, 2008).

But it is difficult to target signaling pathway because LSCs arise from normal stem cells and they share same signaling pathway of normal stem cells (Saito *et al.,* 2010).

Now days plant have been considered as natural source of anti-cancer drugs and play a significant role in the treatment of many cases of cancer types.

Citrullus colocynthis L. from family *Cucurbitacea* is widely naturalized in desert areas including Sudan and well known in Sudan for traditional medicinal plant. *C. colocynthis* known to possess many pharmacological effect including anti-bacterial, anti-fungal and anti-cancer activities (Sawaya *et al.,* 1986) (Marzuk *et al.,* 2010).

The major phytochemical compounds in these plant such as cucurbitacins, glycosides,alkaloids, flavanoids and tannins, traditionally the plant used as anti-diabetic (Shi *et al.,* 2014) (Tannin-Spitz *et al.,* 2001).

Several natural compounds was found to inhibit cancer stem cells as salinomycin, curcumin, sulfaraphane vitamin D (Wang, 2011).

Flowcytometry is widely technique used in all aspects of screening new drugs and it can bave away for drug testing .It was the first time to use such technique in Sudan for estimate and culture LSCs for short term culture.

METHODOLOGY

Material

Drug and chemicals

All chemicals were purchased from local suppliers, human serum albumin 10% and anti-cancer drug vencrstine 1 mg/ml were obtained from hospital of National Cancer Institute, University of Gezira, Sudan. Biomarkers, cluster of differnttiation (CD) where obtained from Flowcytometery Centre Khartoum Sudan .

Plant collection

Citrullus colocynthis (L.) fruits and pulp were collected from Sudan, Gezira State, Wad Medani town, after rainy season, the plant authencuted by National Institute for Promotion of Horticultural Exports, University of Gezira, Sudan by Prof. Mohamed Taha Yousif.

Preparation of plant material

Fruit pulp of plant *C. colocynthis* were dried at room temperature and ground to fine particles by using motor and bistle and kept for further analysis.

Plant Extract

10 gram of dried powder of fruit pulp of *C. colocynthis* (L.) was suspended in 100 ml of ethanol 80% at $37C^0$ for 72 hours. The solvent was evaporated using rotary-evaporator at $42C^0$. The crude extract was then dissolved in ethanol and kept at 20^0C for further use.

Phytochemical screening

Qualitative analysis were carried out for major compounds of *C. colocynthis* that showed anti-cancer activities according to literature data.

Determination of cucurbitacins were carried out by standard method (Attarad and Scicluna-Spiter, 2001). Glycosides, flavanoids and saponin were described by standard method of analysis (Harbone, 1998). Alkaloids subjected to assay according to (Sabri *et al.* 1973).

Selection of patients

In this study three patients entered this trial according to specific criteria. All patients given written informed consent, and all procedures were performed upon the approval of Ethical Committee of Ministry of Health in Gezira State, Sudan. The study involves participants newly diagnosed AML and ALL young and old patients with highly primary bone marrow blast cells at investigation

and this study is short term-culture for six days *in vitro*.

Collection of native bone marrow

Samples of bone marrow were aspirated under general anesthesia from suspected patients at investigation and all procedures of isolation cells were performed by protocol of density separation established by, Boyum (1976).

Cell culture

Pure primary native human bone marrow material after collection and processing were cultured in human serum albumin 10% at $37C^0$ CO_2 humidified incubator.

Immunophenotypic profile at diagnosis

The immunophenotypic profiles at diagnosis aimed to confirm that patients samples were leukemic cells in origin in the bone marrow compartment, and count the percentage of CD34+ and CD45- cells as immature cells .

Protocol assessment anti-LSCs activity

Bone marrow material with confirmed diagnosis highly blast cells of leukemia were subjected for experiments after checking viability of cells using 7.A.A.D dyes. Bone marrow with density of 1 x 10^6 cells/ml were seeded in tissue culture tubes with human albumin serum 10% maintained with buffer solution.

Anti- cancer agent vencrstine 1 mg/ml was diluted at concentration of 0.5 mg/ml as positive control, while ethanolic extract of fruit pulp *C. colocynthis* at concentration of 10 mg/ml induced in cell culture. The control negative experiments were performed with same protocol and conditions of culture tubes without adding any treatment.

Cell viability test

7-Aminoactinomycin D (7.AAD) dyes used to stain dead cells or late apoptotic cells according to cells membrane integrity (Zemburski *et al.*, 2012). And all samples were tested according to protocol of manufacturer company of flowcytometery (Beckman Coulter 500).

7.A.A.D. is cell impermeable stain , it has been used to label dead cells with damaged cell membrane.

Flowcytometery analysis

All tests were assessed by flowcytometery according to manufacturers protocol of company.
Company : Beckman Coulter FC 500
Origin **:** Miami, FL, USA
Running protocol **:** 7AAD/CD45.
The stem cells were identified based on CD34/ high , and CD45/blow .

Statistical analysis

The flowcytometery"Beckman Coulter FC 500" provides statistical information and it is incorporates with analytical software that helps for rapid analysis and reporting of results.

All data analyzed and recoded using software AM14083 Version CXP 2.2.

RESULTS

Ethanolic fruit pulp extract of *C. colocynthis* screened for detection of cucurbitacins, alkaloids, glycosides, flavanoids, and saponin according to standard method of analysis. The results of phytochemical analysis revealed the presences of cucurbitacin alkaloids, glycosides, flavanoid and saponin in ethanolic fruit pulp extract of *C. coloctnthis plant* (Table 1).Several studies have reported the anti-cancer effect of these compounds (Hussain *et al.*, 2013).The phytochemical conducted on fruit pulp extract of colocynthis plant for detection of chemical constituents which are known to exhibit pharmacological activities .

Table 1. Qualitative analysis of fruit pulp *citrullus colocynthis*

Phyto chemicals	Presence	Tests
Cucurbitacins	+++ (high)	Burchards test
Alkaloids	++(moderate)	Mayers test
Glycosides	+++(high)	Legals test
Flavanoids	++(moderate)	Lead acetate test
Saponin	+(low)	Foams test

Tannins	± (absent)	Ferric chloride

All samples were collected after obtaining the written informed consent from patients
or parents and all procedures were performed upon the approval of Ethical Committee of Ministry of Health, Sudan (Table 2).

Table 2: characteristic of patient's Immunophenotyping for patient's sample (1)

Sa	Patients	source	Sex type	age	diagnosis	subtype	Blast cell	Blast cell after purification
Sample1	Patient1	Bone marrow	male	4 years	AML	M2	65.5%	96.1%
Sample2	Patient2	Bone marrow	male	67 years	AML	M2	78.7%	97%
Sample3	Patient3	Bone marrow	female	9 years	ALL	Early pre-all	67.4%	93.8%

The immunophenotypic profile at diagnosis for patient's sample 1 demonostrated the immature size of population 68.5% (table 2) and it was positive for myeloid markers MPO, CD13,CD33, while negative for CD64, CD11c and CD14 . B-cell and T-cell markers were negative (cCD79a and cCD3) .
Immunophenotype compatibale with AML ,with maturation (M2) .

Immunopheno typing for patient's sample (2)

The immunophenotypic for patient's sample 2 showed the immature percentage 78.7% (table 2) and it was positive for CD34 and HLA-DR . It was positive for myeloid markers MPO, CD33 , while negative for CD13 ,CD64, CD11c and CD14 .B-cell and T-celll markers were negative (cCd79 and cCD3).
The immunophenotype compatible with AML with maturation M2 .

Immunophenotyping for patient's sample (3)

The immature for patient's sample 3 was 57.4% (table 2) .The immunophenotype was positive HLA-DR (Strong) and CD34. Immature population was positive for B-cell markers :cCD79a, CD19 ,CD10 (Strong) , while negative for immunoglobulins (Kappa and Lambda) .Myeloid and T-cell markers were negative (MPO,CD13,CD33 and Ccd3).
Immunophenotype compatible with B-cell Acute Lymphoblastic leukemia (Early pre B-ALL

Patient's Sample (1)

Patients 1 represented sample one was male 4 years old newly diagnosed AML subtype M_2 with highly primary blast cells aspirated from bone marrow 65.5% and after purification the blast cells was 97% after treated sample 1 with fruit pulp extract was occurred significant reduction of viable leuckemic stem cells after 3 days and 6 days of incubation period gave 5.2% and 13.5% respectively.

Whereas control positive scored 1.6% and 2.3% respectively. No any significant change observed in culture cell tube of control negative (Table 3 and Figure 1).

Table 3. Percentage of dead stem cells respect to control positive and control negative for sample 1

Treatment	Day1	Day3	Day6
C.colonythis	1.20%	5.20%	13.90%
Control positive	1.20%	1.60%	2.30%
Control negative	1.20%	1.20%	2.20%

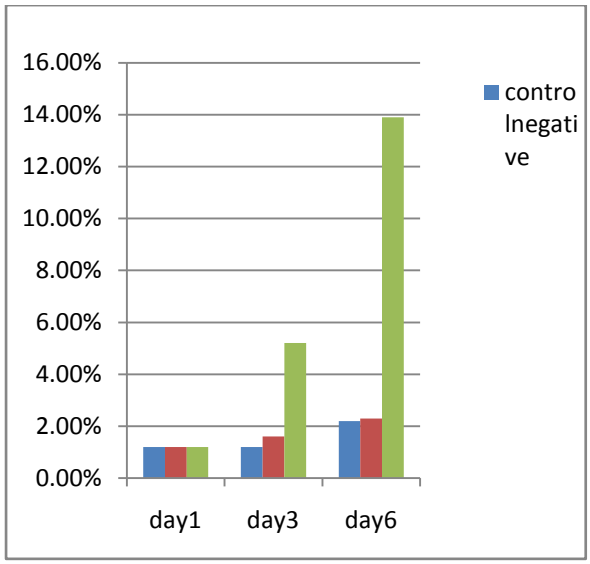

Figure 1. Percentage of dead stem cells respect to control positive and control negative for sample 1

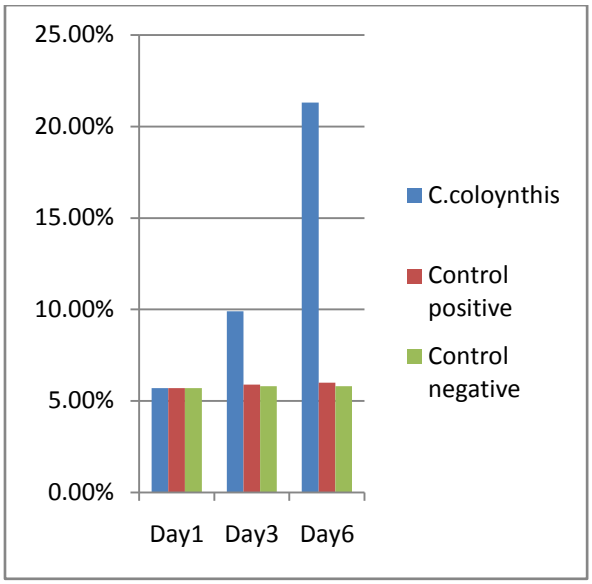

Figure 2. Percentage of dead stem cells respect to control positive and control negative for sample 2

Patient's Sample (2)

Patients 2 represented sample 2 was male 67 years old newly diagnosed AML subtyoe M2 with inicial blast cells 7.8.7% after purification the blast cell was 96.1% after treated sample 2 with fruit pulp extract 3 days and six days it was noticed remarkable inhibition of LSCs compared to control positive and negative control. Treated sample with fruit pulp extract gave 9.9% and 21.3% respectively, whereas positive control gave 5.97% and 6.0% dead cells respectively. No change observed of dead stem cells in control negative (Table 4 and Figure 2).

Table 4. Percentage of dead stem cells respect to control positive and control negative for sample 2

Treatment	Day1	Day 3	Day 6
C.coloynthis	5.7%	9.90%	21.30%
Control positive	5.7%	5.90%	6%
Control negative	5.7%	5.80%	5.80%

Patient's Sample (3)

Patients 3 represented sample 3 was female 9 years old newly diagnosed (ALL) subtype early pre-B-ALL with blast cell 67.4% after purification the blast cell was 93.7%. After 3 and 6 days of treatment sample with fruit pulp extract was observed strongly inhibition of live LSCs relative to control positive. Treated sample with extract fruit pulp scored 24.6% and 29.85 dead cell respectively.

When control positive gave 1% and 6% respectively. No change was noticed cell culture tube of control negative (Table 5 and Figure 3).

Table 5. Percentage of dead stem cells respect to control positive and control negative for sample 3

Treatment	Day1	Day3	Day6
C.coloynthis	0.8%	24.6%	29.8%
Control positive	0.8%	1%	6%
Control negative	0.8%	0.8%	0.8%

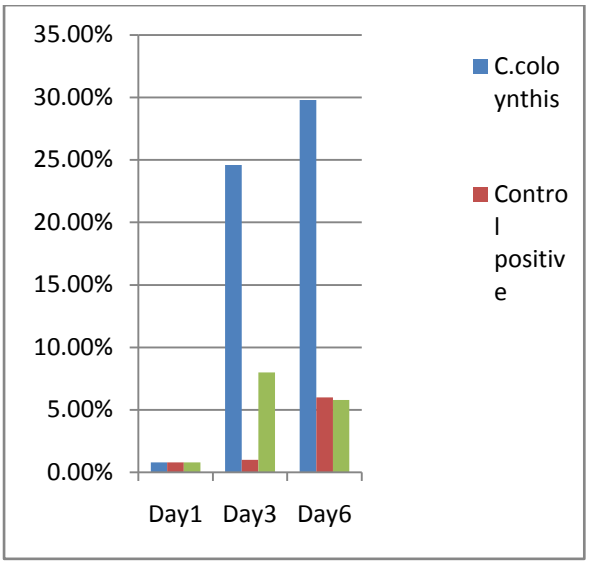

Figure 3. Percentage of dead stem cells respect to control positive and control negative for sample 3

DISCUSSION

In this study the higher percentage of primary native blast bone marrow material were selected and aspirated from newly patients at investigation suggesting a possible enrichment of CD_{34}+CD_{45}- stem cells among suspected patients. Experiments were started after successful collection and processing of native bone marrow material according to protocol of density gradient.

This experiments based on expression of biomarker CD_{34} plus CD_{45} on samples of different leukemic types and subtypes to determine the effect of the extract fruit pulp of *C. colocynthis* on LSCs at concentration of 10 mg/ml dose 30µ/ml with density 1 x 10^6 cell/ml relative to classical anti-cancer vencrstine at concentration 0.5 mg/ml dose of 30µ /ml with density 1 x 10^6 cell/ml using flowcytometric method of analysis according to protocol cell viability test (7.A.A.D) as short term culture for six days of incubation.

Vencrstine 0.5 mg/ml represented as control positive whereas cell culture without treatment represented as negative control.

Data analysis was showed significant differences among treated samples and control samples, fruit pulp extract gave comparable results at 3 and 6 days of incubation period of culture relative to vencrstine 0.5 mg/ml and untreated samples.

Highly significant ($P \leq 0.001$) among samples fruit pulp extract gave 13.5%, 21.3% and 29.8% for sample 1, 2 and sample 3 respectively. Whereas vencrstine gave mortality rates of 2.3%, 6% and 6% for sample 1, 2 and 3 respectively. When no any significant change was observed in culture tubes of control negative.

The results showed that the anti-cancer drug vencrstine at concentration of 0.5 mg/ml do not work well and have lower effect on leukemic stem cells comparing to fruit pulp extract of *C. colocynthis* at concentration 10 mg/m; at the same doses 30µ /ml. It was noticed remarkable inhibition of LSCs in sample number 3 with ALL type of leukemia compared to sample 1 and sample 2 with AML types of leukemia.

Rare studies were conducted on LSCs and the results of this experiments are not routinely a viable at investigation to capture the factors determine the behavior , of stem cells our study included small numbers of patients because difficult to collect native bone marrow material from donors.

Bone marrow services as ideal source for studying stem cells and the biggest obstacles to collect bone marrow such as very painful, post infection risk and limitation of donors.

LSCs co-exist in the bone marrow with association between normal stem cells and there are sharing same signaling pathway representing significant challenge to target LSCs.

The LSCs may persist after treatment and drive new more aggressive form of leukemia leading to relapse (Vetrie *et al.*, 2020).

Our findings suggest that fruit pulp extract from *C. colocynthis* induced apoptosis against stem cells of leukemia with no significant differences between both types of leukemia AML and ALL and their subtypes.

However we did not obtained completely inhibition of LSCs in treated samples, we concluded that further experiments in variety concentration for extract fruit pulp may induce complete inhibition of LSCs.

Further studies are required to identify bioactive compounds in fruit pulp extract and their pharmacological effect on leuckemic stem cells for therapeutic intervention.

In the end with hope to help future studies to develop more effective treatment for leukemia from natural compounds to eliminate LSCs.

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